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Structure and Function of the Molluscan Myoactive Tetradecapeptides

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ABSTRACT—Effects of myoactive tetradecapeptides, *Achatina* excitatory peptide 2 and 3 (AEP2 and AEP3) and *Fusinus* excitatory peptide 4 (FEP4), on several molluscan muscles and neurons were investigated. In the penis retractor and radula retractor muscles of *Achatina fulica* (pulmonate), the three peptides enhanced the tetanic contraction elicited by nerve stimulations. The order of potency was AEP2>AEP3>FEP4, although the effects of AEP3 and FEP4 on the radula retractor were somewhat irregular. AEP2 also induced rhythmic bursts of activity in the buccal ganglionic neuron B4 known as a cholinergic motoneuron of the radula retractor. In the radula protractor and retractor muscles of *Fusinus ferrugineus* (prosobranch), FEP4 was most potent in enhancing the contraction. The enhancement was greater in the protractor than in the retractor. It was suggested that myoactive tetradecapeptides modulate mainly the cholinergic transmission in molluscan muscles.

INTRODUCTION

It has been known that the contraction of molluscan muscles is regulated by multiple bioactive substances including neuropeptides [11]. In the African giant snail *Achatina fulica* (pulmonate, mollusc), more than twenty neuropeptides have been purified from the ganglia and hearts [8], and the function of some of them has already been investigated [1, 5]. Two bioactive tetradecapeptides which are highly homologous in structure were isolated from the ganglia of this snail [10]. Further, they appeared to be very close to another tetradecapeptide purified from the ganglia of a prosobranch *Fusinus ferrugineus*. During the process of purification of these peptides, they were found to be active in enhancing the contraction of a few muscles of *A. fulica* [10]. They were named myoactive tetradecapeptides (MATPs) [10].

In the present study, actions of MATPs on several molluscan muscles and neurons were examined and the mechanisms of their action on the neuromuscular systems were studied.

MATERIALS AND METHODS

Two *Achatina* tetradecapeptides and a *Fusinus* tetradecapeptide were isolated from the ganglia of each animal by using the penis retractor muscle of *A. fulica* and the radula retractor muscle of *F. ferrugineus* as a bioassay system, respectively. These isolation procedures have been described previously [10].

Peptides used in the present experiments were synthesized by a solid-phase peptide synthesizer and purified by HPLC. Their structures were confirmed by amino acid sequence analysis and fast atom bombardment mass spectrometric (FAB-MS) measurement.

Bioactivities of the synthesized peptides were examined on the penis retractor muscle, the radula retractor muscle and the buccal ganglionic neurons of *A. fulica*, and the radula protractor and retractor muscles of *F. ferrugineus* and of *Rapana thoma-*

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siana (prosobranch). Physiological saline used for the preparation from *A. fulica* consisted of (mM): NaCl 61.0, KCl 3.3, $CaCl_2$ 10.7, $MgCl_2$ 13.0, glucose 5.0 and Hepes-NaOH 10.0 (pH 7.5). In some experiments, saline containing 65 mM $MgCl_2$ (high-Mg^{2+} saline) was used, which was prepared by merely adding $MgCl_2$ to normal saline. Composition of artificial seawater used for the preparation from *F. ferrugineus* and *R. thomasiana* was as follows (mM): NaCl 475.0, KCl 10.0, $CaCl_2$ 10.0, $MgCl_2$ 20.0 and Tris-HCl 10.0 (pH 7.8).

Stimulation of these muscles and recording of the tension were made according to the method previously described by Muneoka and Twarog [9]. Electrical activities of the neurons were recorded by the conventional intracellular microelectrode method [13].

RESULTS

Two *Achatina* peptides were provisionally termed *Achatina* excitatory peptide 2 (AEP2) and 3 (AEP3), and a *Fusinus* peptide was termed *Fusinus* excitatory peptide 4 (FEP4) [10]. The structures of these peptides are as follows:

AEP2: Gly-Phe-Arg-Gln-Asp-Ala-Ala-Ser-Arg-Val-Ala-His-Gly-Tyr-NH_2

AEP3: Gly-Phe-Arg-Gly-Asp-Ala-Ala-Ser-Arg-Val-Ala-His-Gly-Phe-NH_2

FEP4: Gly-Phe-Arg-Met-Asn-Ser-Ser-Asn-Arg-Val-Ala-His-Gly-Phe-NH_2

In the present paper these terms will also be employed.

Figure 1A demonstrates effects of three kinds of peptides on the tetanic contraction of the penis retractor muscle of *A. fulica*. The contraction was

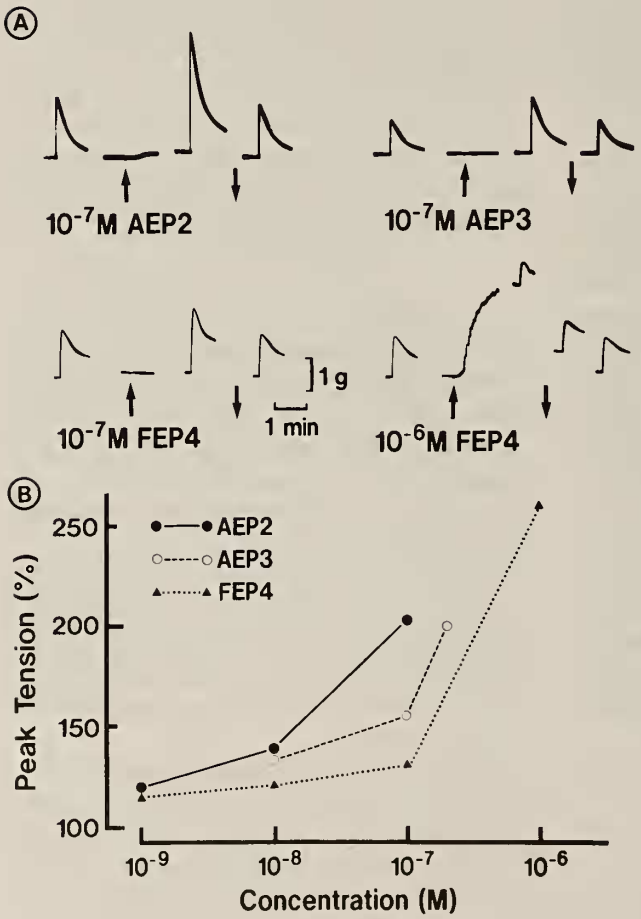


FIG. 1. Effects of three peptides on the tetanic contraction of the penis retractor muscle of *A. fulica*. A. The muscle was stimulated with a train of electrical pulses (0.8 msec, 10 V, 40 Hz) for 1 sec at 10 min intervals. Peptide was applied 8 min before applying the stimulation (upward arrow), and washed out immediately after the recording (downward arrow). B. Dose-response relationships of three peptides. Peak tension was expressed as percent of the control tension.

evoked by applying a train of electrical pulses (0.8 msec, 10 V, 40 Hz) for 1 sec to the muscle. AEP2 remarkably enhanced the contraction with a threshold concentration of 10^{-9} M, and at concentrations more than 10^{-7} M it directly induced a contraction. AEP3 and FEP4 produced similar effects on the penis retractor muscle, although they were slightly less potent than AEP2. Figure 1B illustrates the dose-response relations of the three peptides. As shown in this graph the order of potency in enhancing the muscle contraction was AEP2>AEP3>FEP4.

To test the site of action of the peptides the following experiments were performed (Fig. 2). After confirming that a train of pulses of short duration (0.8 msec, 10 V, 40 Hz) produced a tetanic contraction in normal saline, it was replaced by high-Mg^{2+} saline. In the latter the contraction by electrical pulses of short duration (0.8 msec) was completely blocked, supporting the assumption that short-duration pulses stimulated the muscle indirectly through intramuscular nerve fibers. When pulse duration was increased to 8.0 msec, however, the tetanic contraction of considerable size could be elicited even in high-Mg^{2+} saline. Such long-duration pulses probably stimulated the muscle fibers directly. AEP2 of 10^{-8} M, which potentiated the tetanic contraction by short-duration pulses in normal saline, could also potentiate the contraction in response to long-duration pulses in high-Mg^{2+} saline. Similarly, AEP3 and FEP4 also enhanced the contraction by long-duration pulses in high-Mg^{2+} saline and the efficiency of the three peptides was about the same.

In experiments shown in Fig. 3, effects of the three peptides on the tetanic contraction of the radula retractor muscle elicited by a train of pulses of short duration (0.8 msec, 10 V, 40 Hz) were examined. AEP2 enhanced the contraction in a dose-dependent manner with a threshold concentration around 10^{-9}-10^{-8} M, and at concentrations of 10^{-6} M or more it directly induced a contraction. However, the effects of AEP3 and FEP4 were quite variable with different preparations, and in some preparations inhibition of contraction during application and potentiation after washing of the peptide were observed (Fig. 3). These results may imply that AEP3 and FEP4 act on multiple nerve fibers of inhibitory and excitatory nature.

In applying several kinds of bioactive amine substances such as acetylcholine (ACh), catecholamines, octopamine, serotonin and histamine on the radula retractor muscle, we have found that only ACh at comparatively lower concentrations

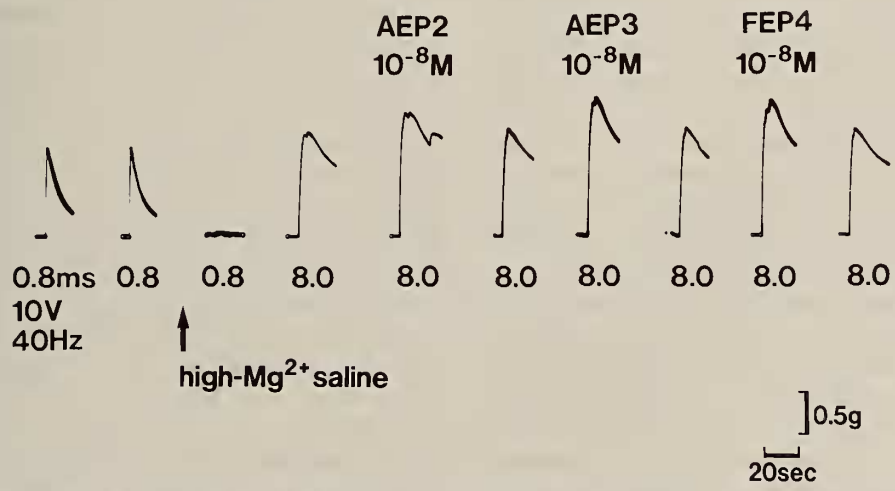


Fig. 2. Effects of three peptides on the tetanic contraction of the penis retractor muscle of *A. fulica* in normal saline and high-Mg^{2+} saline. The muscle was stimulated with a train of short-duration pulses or long-duration pulses at 10 min intervals. Soon after recording the second contraction, high-Mg^{2+} saline was applied (upward arrow). Procedures for applying peptides were the same as in Fig. 1.

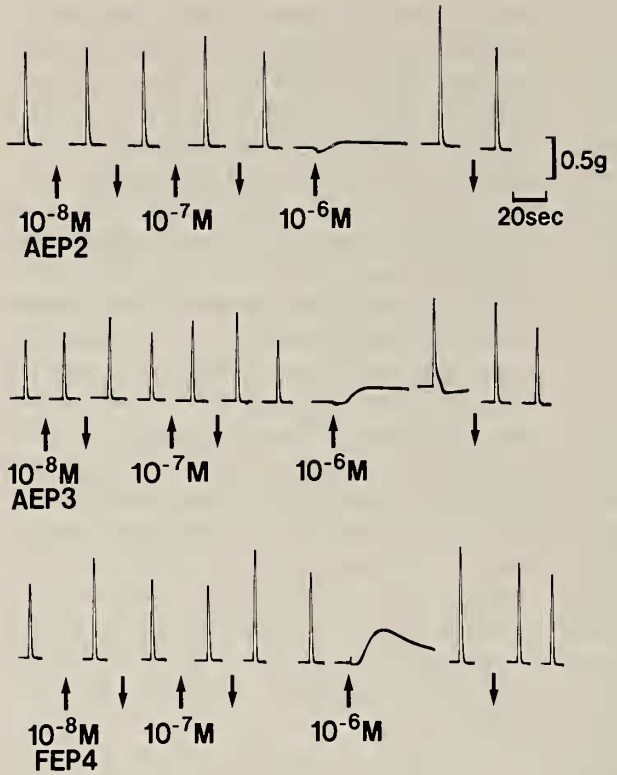


FIG. 3. Effects of three peptides on the tetanic contraction of the radula retractor muscle of *A. fulica*. Procedures for stimulating the muscle and applying peptides were the same as in Fig. 1.

elicited a contraction. Further, an identified motoneuron (B4) of the radula retractor muscle has been shown to be cholinergic [13]. Thus, the modulatory effects of peptides on the contraction in response to ACh were investigated. Figure 4A shows contractions evoked by applying ACh during the period indicated by a horizontal line under each record. AEP2 potentiated the ACh-evoked contraction in a dose-dependent manner, and AEP3 and FEP4 also showed similar effects. The order of efficiency was AEP2>AEP3>FEP4 as shown by dose-response curves (Fig. 4B).

In molluscan neuromuscular junctions, propantheline has been shown to be a cholinergic blocking agent [12, 13]. In the experiments shown in Fig. 5A and B effects of propantheline on the contraction of radula retractor muscle elicited by ACh and by electrical stimulation were compared. ACh-evoked contraction was extremely depressed by propantheline at a concentration of 10^{-5} M and was completely blocked at 10^{-4} M (Fig. 5A). However, the contraction in response to electrical stimulation was inhibited by only 30% of the control during application of 10^{-4} M propantheline. To demonstrate that short-duration pulses (0.6 msec) selectively stimulated the intramuscular nerve fibers the preparation was immersed in high-Mg^{2+} saline. Stimulation of short-duration pulses almost failed to evoke the contraction, but that of long-duration pulses (6.0 msec) elicited a contraction of considerable size, supporting the foregoing assumption. Propantheline of 10^{-4} M did not depress the contraction by long-duration pulses in high-Mg^{2+} saline. From these experiments, the contraction elicited by short-duration pulses during application of propantheline in normal saline is considered to be evoked by the stimulation of nerve fibers other than cholinergic ones. Will this contraction be modulated by peptides? Experiments shown in Fig. 5C were performed to answer this question. AEP2 of 10^{-7} M enhanced the contraction elicited during application of propantheline by only about 14%, in contrast to the contraction without propantheline

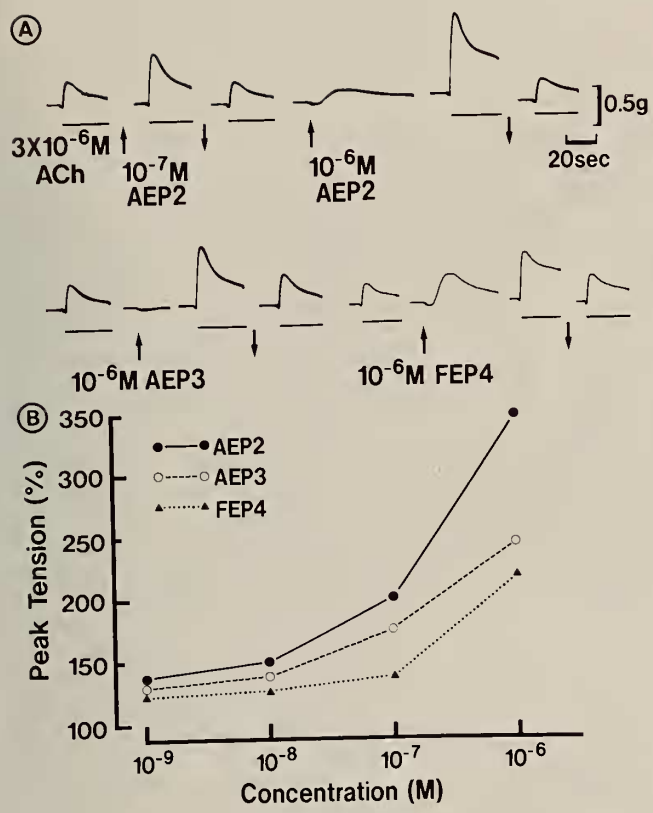


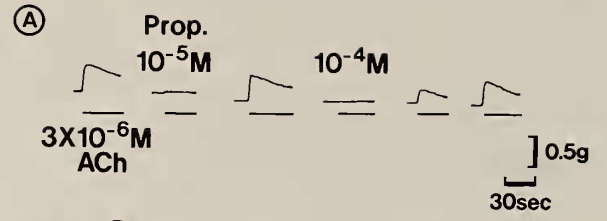
FIG. 4. Effects of three peptides on the contraction of the radula retractor muscle of *A. fulica* induced by ACh. A. ACh was applied during the period shown by a horizontal line under each record. Procedures for applying peptides were the same as in Fig. 1. B. Dose-response relationships of three peptides. Peak tension was expressed as percent of the control tension.

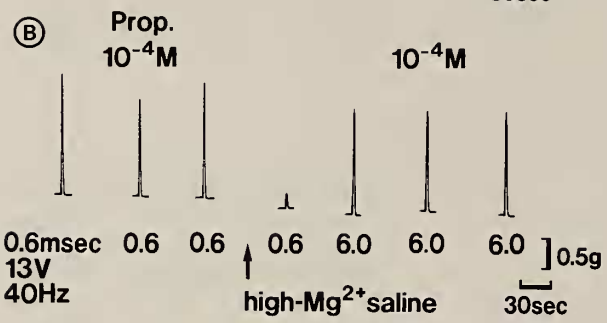
which was enhanced by about 46% by applying the same concentration of AEP2. This seems to imply that AEP2 enhances the contraction of the radula retractor muscle by acting mainly on the cholinergic transmission.

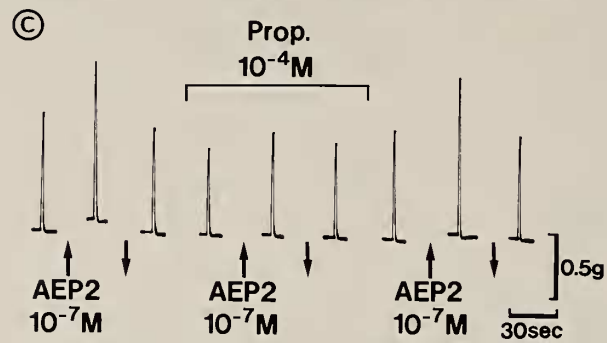
Among the identified neurons in the buccal ganglia of *A. fulica,* six pairs have been known as excitatory motoneurons for the radula retractor muscle [13, 14]. Figure 6 shows effects of the peptides on such identified neurons B1 and B4. AEP2 induced rhythmic bursts of activity in both neurons (Fig. 6A), and AEP3 also elicited several bursts, although they were rather irregular (Fig. 6B). However, FEP4 had no significant effects on these neurons.

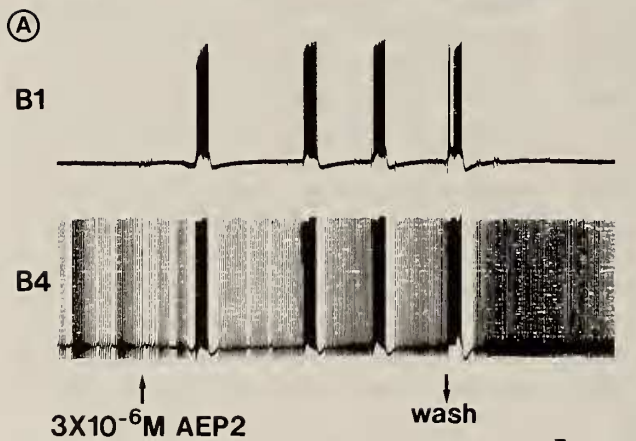
In experiments shown in Fig. 7, effects of the three peptides on the twitch contraction of the radula protractor and retractor muscles of *Fusinus ferrugineus* were tested. In these muscles FEP4 showed stronger effects than AEP2 or AEP3 in contrast to their effects on *Achatina* muscles and neurons. FEP4 enhanced the contraction in a dose-dependent manner with a threshold concentration of 10^{-10} M, and at concentration of 10^{-8} M or more it directly induced a contraction. Similar tendency of effects was observed in AEP2 and AEP3, with AEP3 having weaker effects than the others. However, the efficiency of the peptides was different between these two muscles; in the radula protractor 10^{-7} M FEP4 enhanced the contraction by more than 150% of the control, while in the retractor the enhancement was less than 30% of the control (Fig. 7). AEP2 and AEP3 also produced stronger enhancing effects on the contraction of the protractor than the retractor.

Similar experiments were performed by using the radula protractor and retractor muscles of *Rapana thomasiana,* since the mode of excitatory transmission has been shown to be different between these muscles, the protractor being mainly cholinergic and the retractor glutaminergic [6, 7]. As demonstrated in Fig. 8, the three peptides









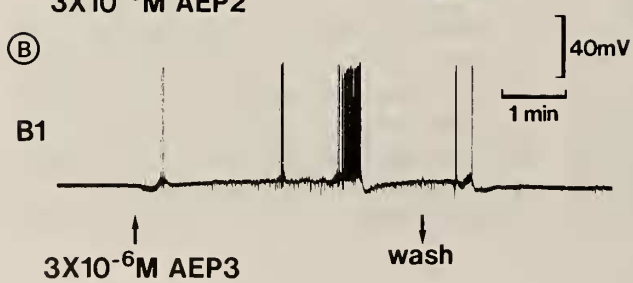


FIG. 5. Effects of propantheline (Prop) on the contraction of the radula retractor muscle of *A. fulica* elicited by ACh (A) and by electrical stimulation (B). Propantheline was applied 8 min before giving ACh or stimulation and washed out immediately after the record. In A, ACh was applied during the period shown by a horizontal line under each record. In B, the muscle was stimulated with a train of short-duration pulses or long-duration pulses at 10 min intervals. Soon after the third record, high-Mg^{2+} saline was applied (upward arrow). C. Effects of AEP2 on the tetanic contraction of the radula retractor muscle of *A. fulica* during and without application of propantheline. The muscle was stimulated with a train of short-duration pulses (0.6 msec, 13 V, 40 Hz) for 1 sec at 10 min intervals. Propantheline was applied 8 min before the fourth record and washed out after the sixth record. Procedures for applying peptide were the same as in Fig. 1.

FIG. 6. Effects of AEP2 (A) and AEP3 (B) on the spontaneous activity of identified neurons B1 or B4 in the buccal ganglion.

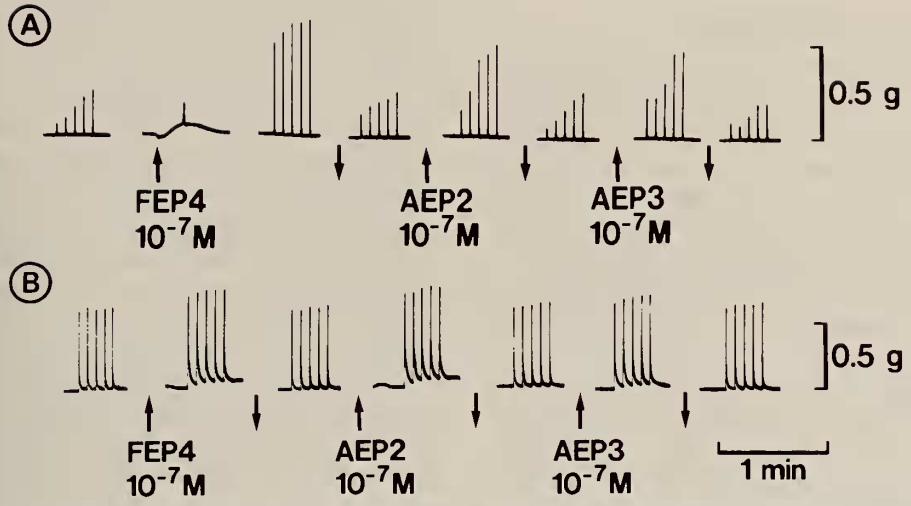


FIG. 7. Effects of three peptides on twitch contractions of the radula protractor muscle (A) and the retractor muscle (B) of *F. ferrugineus*. The muscle was stimulated with a train of electrical pulses (2 msec, 15 V, 0.2 Hz, 5 pulses) at 10 min intervals. Procedures for applying peptides were the same as in Fig. 1.

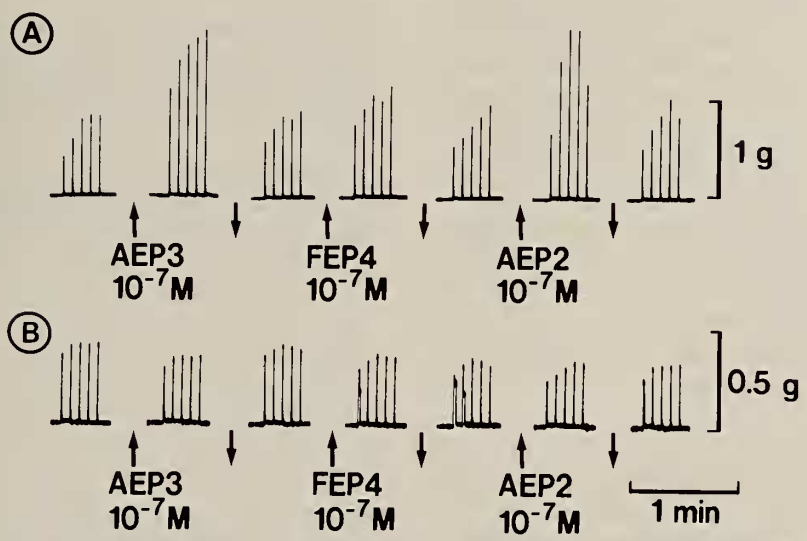


FIG. 8. Effects of three peptides on twitch contractions of the radula protractor muscle (A) and the retractor muscle (B) of *R. thomasiana*. The muscle was stimulated with a train of pulses (1 msec, 15 V, 0.2 Hz, 5 pulses) at 10 min intervals. Procedures for applying peptides were the same as in Fig. 1.

strongly increased twitch contractions of the radula protractor, but they did not show any enhancing effects on the contraction of the retractor. Finally, effects of the peptide AEP3 on the contraction evoked by application of ACh or glutamate were compared between these muscles. In the radula protractor 10^{-7} M AEP3 enhanced ACh-induced contraction prominently, whereas in the retractor it showed no significant effects on ACh-induced as well as glutamate-induced contractions (data not shown).

DISCUSSION

From results obtained in the present experiments the functional mechanisms of myoactive tetradecapeptides (MATPs: AEP2, AEP3 and FEP4) were studied. In the penis retractor muscle of *A. fulica,* the three peptides enhanced the tetanic contraction elicited by nerve stimulations and the order of potency was AEP2>AEP3> FEP4. On the other hand, the enhancing effect of the three peptides on the contraction evoked by muscle stimulations was about the same. These results may well be explained by assuming that actions of the three peptides on the presynaptic sites are different. Molluscan muscles generally receive multiple innervations [2, 11], and it is possible that the three kinds of peptides showed different modulatory actions on the nerve fibers.

In the radula retractor muscle of *A. fulica,* AEP2 enhanced the tetanic contraction in a dose-dependent manner with a threshold concentration as low as 10^{-9} M, suggesting the possibility that this peptide acts physiologically in the regulation of contraction of this muscle. AEP2 also induced rhythmic bursts of activity in the buccal ganglionic neuron B4 which is known as a cholinergic motoneuron of the radula retractor [13]. Furthermore, from the pharmacological examination using propantheline, AEP2 was suggested to act mainly on the cholinergic transmission. Therefore, it is considered that AEP2 regulates contraction of the radula retractor muscle by acting excitatorily on the ganglionic neuron(s) as well as on the muscle.

There are some instances that a kind of peptide regulates the muscle contraction by acting both on the central nervous system and the periphery. Achatin-I isolated from *A. fulica* increased the heart activity by acting excitatorily on the cardio-excitatory neuron PON and on the ventricle of this snail [1]. On the other hand, FMRFamide was shown to act inhibitorily on the central neurons and excitatorily on the myocardium to regulate the heart beat [3].

In the radula muscles of *F. ferrugineus,* on the other hand, FEP4 isolated from this animal showed the most potent activity of the three peptides. FEP4 showed strong enhancing effects on the twitch contraction of the protractor with a threshold concentration of 10^{-10} M, suggesting that FEP4 physiologically regulates the contraction of the radula protractor muscle. It is noteworthy that in *F. ferrugineus* and also in *R. thomasiana* the three peptides showed strong enhancing effects on the contraction of the radula protractor but slight or no effects on the radula retractor. Our previous studies have shown that in the radula protractor of *R. thomasiana* the principal excitatory neurotransmitter is ACh, whereas in the retractor it is glutamate [6, 7, 12]. The same appears to be true in the radula muscles of *F. ferrugineus* (Muneoka, unpublished data). The present results suggest that the three peptides enhanced the contraction of the radula protractor by modulating the transmission mediated by ACh. It is probable that in general MATPs act mainly on the cholinergic transmission in molluscan muscles. Several results obtained in the present experiments appear to support this notion.

We found that the amino acid sequence of a peptide allatotropin purified from the tobacco hornworm *Manduca sexta* [4] is significantly homologous to that of MATPs as shown below.

AEP2	G F R Q D A A S R V A H G Y amide
AEP3	G F R G D A A S R V A H G F amide
FEP4	G F R M N S S N R V A H G F amide
Allatotropin	
	G F K * N V E M M T A R G F amide

Although allatotropin is not a 14-residue peptide but a 13-residue peptide, it shares 36% of residues with AEP3 and 43% with FEP4. Particularly, the N- and C-terminal dipeptide moieties of allatotropin are the same with those of the three molluscan peptides, except the C-terminus of AEP2. Since we have not yet examined the effects of allatotropin on molluscan muscles, the structure-action relationships are not known. However, these results are considered to suggest that MATP-related peptides are distributed not only in molluscs but also in other phyla including arthropods.

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